UNDERSTANDING THE RELATION BETWEEN RNASES H AND RETROTRANSPOSITION ACTIVITY IN THE CONTEXT OF THE AICARDI-GOUTIÈRES SYNDROME

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UNDERSTANDING THE RELATION BETWEEN RNASES H AND RETROTRANSPOSITION ACTIVITY IN THE CONTEXT OF THE AICARDI-GOUTIÈRES SYNDROME

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For my grateful people

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LIST OF SYMBOLS AND ABBREVIATIONS

/	per
℃	degrees Celsius
α	alpha
%	percentage
Δ	used for annotation of deleted genes
cDNA	complementary DNA
DNA	deoxyribonucleic acid
ds	double-stranded
g	gram
hr	hour
mg	milligram
ml	milliliter
RNA	ribonucleic acid
SC-Ura$^-$	synthetic complete medium lacking uracil
SC-His$^-$	synthetic complete medium lacking histidine
GalUra$^-$	synthetic complete medium lacking uracil + 2 % galactose
Ty	transposons of yeast
RNase H	ribonuclease H
AGS	Aicardi-Goutières syndrome
S. cerevisiae	*Saccharomycess cerevisiae*

IFN	interferon
VLP	virus-like particle
mRNA	messenger RNA
AI	artificial intron
kanMX4	kanamycin cassette
hygMX4	hygromycin cassette

SUMMARY

Ribonucleases (RNases) H1 and H2 are endonucleases that hydrolyze the RNA strand of RNA-DNA hybrids forming at the chromosomal level as well as extra-chromosomal hybrids. Extra-chromosomal RNA-DNA hybrids can frequently occur in cells as intermediate structures in the process of reverse transcription and generation of cDNA by retrotransposition. It is known that mutations in RNase H2 are found in Aicardi-Goutières syndrome (AGS) patients. AGS is a rare but severe immune-mediated neurodevelopmental disorder. Currently, the mechanism by which defects in RNase H2 cause AGS is still unclear. We hypothesized that defects in RNases H, including those associated with AGS can trigger the accumulation of extra-chromosomal RNA-DNA hybrids. Thus, we speculate that increased stability of such free RNA-DNA hybrid structures could be a likely trigger for stimulating the autoimmune system, mimicking a viral infection in AGS patients. RNase H2 protein subunits of human and yeast *Saccharomyces cerevisiae* RNase H2 proteins have conserved amino acid sequences. Based on the similarity between human and yeast RNase H2, we thought to utilize *S. cerevisiae* as a research model to generate and study several AGS-related mutants. Initially, we set up an assay to detect retrotransposition activity in the budding yeast by introducing a recombinant DNA which includes a Ty1 retrotransposable element fused to an inactive *his3* marker gene. To test whether the retrotransposition assay works in our yeast strains, we treated yeast cells with phosphonoformic acid (PFA) or knocked out *DBR1* gene coding for the RNA lariat debranching enzyme. Both approaches strongly reduced the frequency of retrotransposition in our strains, demonstrating that the system was working as expected. Next, we examined whether yeast cells with defective forms of

RNases H or AGS-orthologous mutants of RNase H2 had altered retrotransposition activity compared with cells with wild-type RNases H. Results showed that the retrotransposition activity was repressed in the absence of both types of RNase H. In addition, AGS-related mutants showed decreased retrotransposition frequencies when RNase H1 was also knocked-out. These findings are relevant to uncover the mechanism of the AGS.

CHAPTER 1

INTRODUCTION

1.1 AICARDI-GOUTIÈRES SYNDROME

In 1984, Aicardi-Goutières syndrome (AGS) was first described by Jean Aicardi and Françoise Goutières, who initially observed that eight children from five families underwent a neurodevelopmental disorder of the central nervous system [1]. In four years, Lebon found that increased levels of antiviral cytokine interferon-α (IFN-α), an integral defense organization of cells against viral infections, was observed in infant patients and attributed to mimicked viral infection [2]. This syndrome is characteristically classified as a congenital and inflammatory disease. Some AGS-inherited infants show abnormal physical and molecular features at birth, such as elevated liver enzymes, thrombocytopenia, and enlarged liver and spleen [3]. In general, symptoms and signs of AGS are seen in few weeks after birth [3, 4]. About 40% of AGS patients suffer from painful and itchy skin lesions on their ears, fingers, and toes **(Figure 1-1)**. AGS is also accompanied by brain dysfunction arising from cerebral calcification white matter abnormalities, and cerebral atrophy. As the result, AGS patients generally have a small head due to slow growth of brain and skull and undergo a phase of inflammation and continuous tissue damage in the central nerve system. Due to the severe impacts on the people with AGS, about 35% of the patients die during their childhood [3, 5, 6].

Figure 1-1. Examples of chilblain lesions seen in Aicardi-Goutières syndrome patients. Adapted from Crow (2008) [31].

It has been known that AGS can be caused by mutations in any of six genes, which are TREX1 (3'→5' exonuclease), SAMHD1 (dNTP triphosphatase), ADAR1 (RNA-editing enzyme), RNASEH2A, RNASEH2B, and RNASEH2C (Subunits of type 2 ribonuclease) [7, 8, 9, 10]. Even though the exact mechanism of causing AGS has not been identified, it is clear that all the six genes are directly or indirectly associated with removal of nucleic acids. Therefore, a failure of removing nucleic acids has been considered as primary cause of the disease. In 2009, Crow and Rehwinkel speculated that an aberrant accumulation of nucleic acids, such as DNAs, RNAs, and RNA-DNA hybrids arising from incomplete nucleic acids removal brings about induction of autoimmunity by IFN-α in absence of any viral factors **(Figure 1-2)** [3, 10].

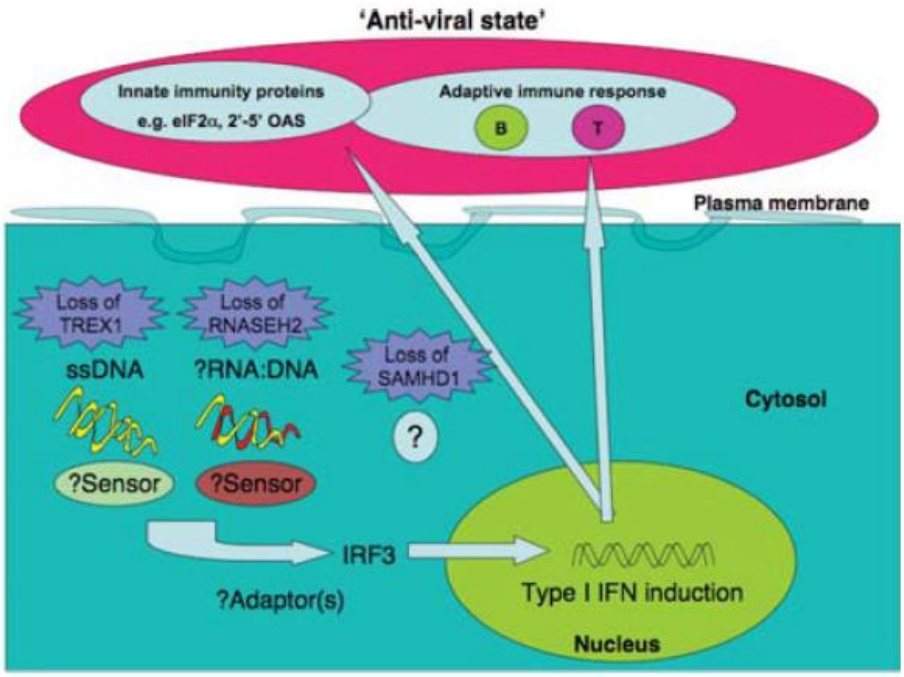

Figure 1-2. Model of AGS outbreak in the absence of AGS-related genes. Adapted from Crow (2009) [3].

The accumulation of nucleic acids might be detected by some unknown sensors, and an induction signal would be transmitted to type I interferon via interferon transcription factor3 (IRF3) so that the brain could be damaged by the activated interferon. To develop treatments for AGS, interrupting each step of AGS-causing pathways was possibly suggested **(Figure 1-3)**.

Despite many efforts in AGS research, any cure has not been discovered yet. However, some treatments with anti-interferon and anti-inflammatory therapies are thought to attenuate of AGS [11].

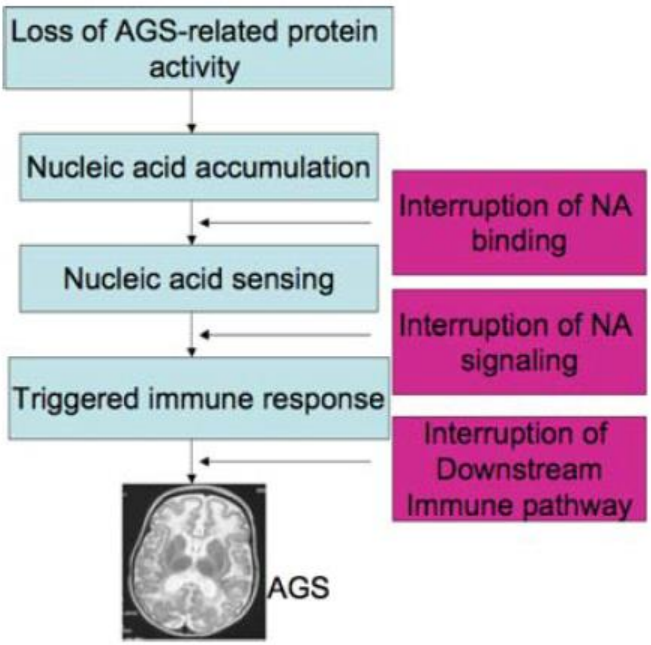

Figure 1-3. Steps in the pathogenesis of AGS which might be discontinued by therapeutic interruption. (NA: nucleic acids). Adapted from Crow (2009) [3].

1.2 RIBONUCLEASE H

Ribonucleases H (RNases H) are a group of endonucleases that hydrolyze the RNA in RNA/DNA hybrids in a non-sequence specific manner [3]. RNases H are classified into type 1 RNases H (or RNases H1) and type 2 RNases H (or RNases H2) according to primary amino acid sequence and biochemical properties on substrate [12]. It is known that RNases H contribute to genome integrity by removing ribonucleotides which are mistakenly incorporated into DNA strands. RNases H1 require at least four ribonucleotides to function appropriately. In contrast, RNases H2 are able to cleave even a single ribonucleotide **(Figure 1-4)** [12, 13]. Most organisms have at least one type of RNases H. In addition, RNases H are indispensable enzymes for multicellular eukaryotes whereas prokaryotes and some single cell eukaryotes do not need the enzymes to survive [12].

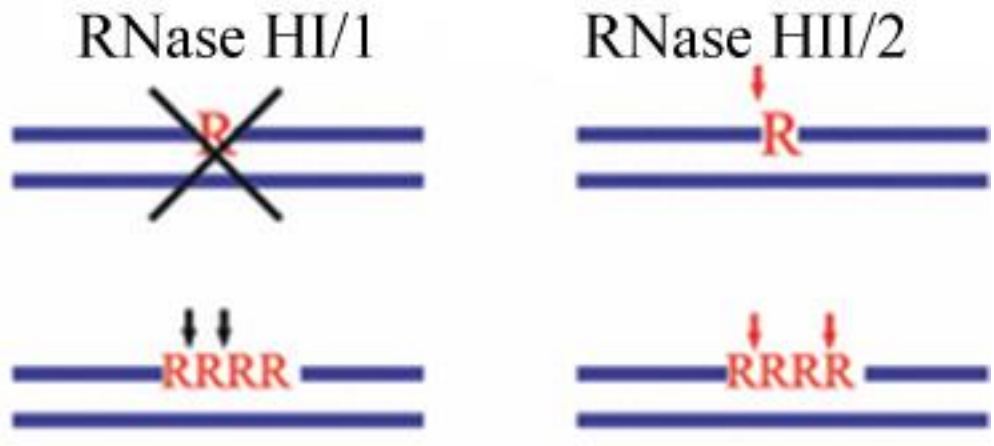

Figure 1-4. Distinct cleavage patterns of RNase HI/1 and RNase HII/2 on different substrates. Adapted from Cerritelli and Crouch (2009) [13].

1.2.1 RNASES H1

RNase H1 is an endonuclease enzyme cleaving the RNA of RNA/DNA heteroduplexes [13]. At least four ribonucleotides are required for this enzyme to function in removal of RNA. RNase H1 plays a critical role in DNA replication, recombination, repair, and trancscription [13, 14]. The N-terminal domain of RNase H1 is referred to as hybrid-binding domain (HBD) [15, 16], which grants more affinity to RNA/DNA hybrids than the same sequence of double-stranded RNA in terms of substrate preference. By forming a strong connection with RNA/DNA duplexes, the HBD aids the catalysis of RNase H domain [17]. In contrast to RNase H2, mutations in RNase H1 do not cause AGS.

1.2.2 RNASES H2

In yeast, RNase H2 (or RNase H35) is another essential repair enzyme, whose main role is the removal of ribonuleotides from DNA. [13]. However, unlike RNase H1, RNase H2 consists of three different subunits: RNase H2A, RNase H2B, and RNase H2C (RNH201, RNH202, and RNH203 in yeast *Saccharomyces cerevisiae*, respectively). RNase H2A, the catalytic subunit, interacts with the other subunits, non-catalytic subunits, to cleave ribonucleotides embedded in DNA duplex [18]. By binding to RNase H2A subunit, RNase H2B and H2C act as a platform to assist the cleavage mechanism [11]. It is also found that RNase H2B includes PCNA-interacting peptide (PIP) to bind proliferating cell unclear antigen (PCNA), which is an indispensable protein for DNA replication/repair as well as Okazaki fragment processing during lagging strand synthesis in Eukaryotes[12, 13]. Type 2 RNases H are able to cleave even a single ribonucleotide mistakenly

incorporated in a RNA-DNA hybrid. In general, this type of RNases H is known to contribute to genome stability by helping DNA repair. However, the damages arising from mutations in RNases H2 can cause severe diseases, such as AGS [3, 13]. It has been proposed that a failure in removing ribonucleotides by RNases H2 induce an aberrant accumulation of RNA/DNA hybrids, which triggers IFN-α upregulation that could damage the brain in the non-viral environment **(Figure 1-5)** [3, 10]. AGS can be caused by mutations in each subunit of RNase H2, especially mutations in RNase H2B among all RNase H subunits are found most frequently in people with AGS. The mechanism causing AGS is still veiled.

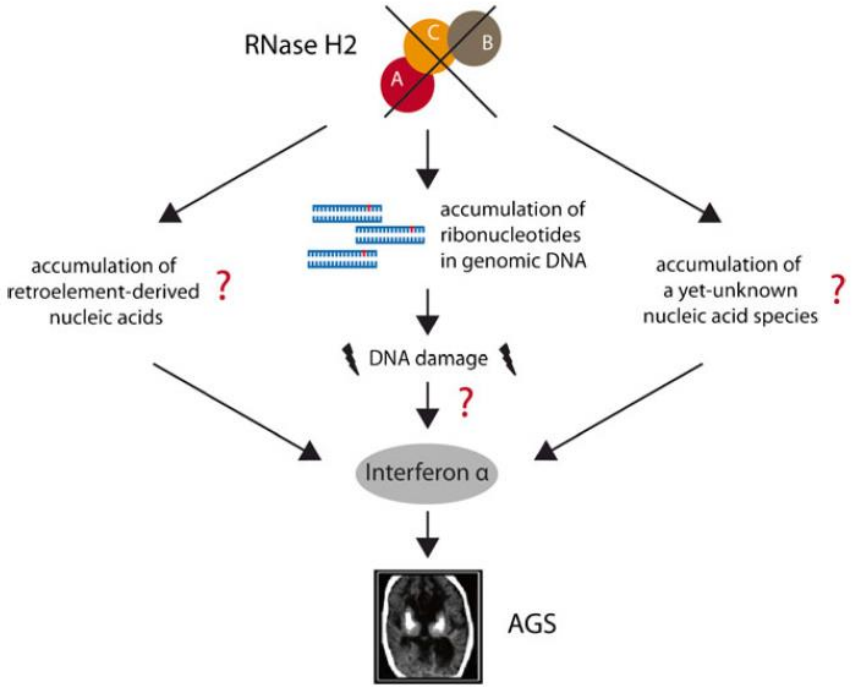

Figure 1-5. A suggested AGS-causing model when RNases H2 are deficient. Adapted from Rabe (2013) [10].

1.3 AGS-RELATED MUTATIONS FOUND IN HUMAN RNASES H2

Crow, Jackson, and Rice research teams revealed that several AGS-related mutations in RNase H2 subunits are commonly found from AGS patients **(Figure 1-6)** [5, 19]. It was suggested that the G37S mutant protein in RNASEH2A lost most RNase H activity due to the substitution nearby the catalytic center. Furthermore, some other mutant proteins are found in the non-catalytic subunits RNASE H2B and RNASEH2C. Surprisingly, the R69W substitution showed reduced RNase H activity like the G37S substitution, but other substituted mutant proteins did not differ from their wild type enzymes. This observation may be explained by the fact that these proteins are involved in RNase H2 stability or interact with other cellular proteins.

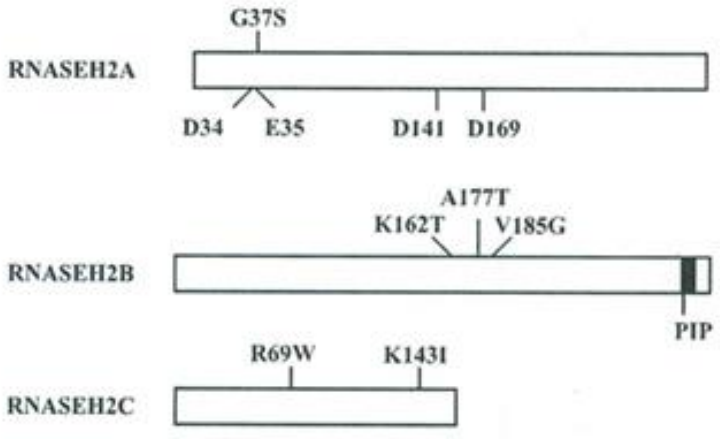

Figure 1-6. A diagram of the three subunits in Human RNase H2. AGS-related mutations are shown on the top of each subunit. Catalysis-involved amino acids are shown on the bottom in RNASEH2A and PIP in RNASEH2B. Adapted from Cerritelli and Crouch (2009) [13].

1.4 SIMILARITY BETWEEN HUMAN AND *S. CEREVISIAE* RNASES H2

There are some conserved amino acid sequences between human and yeast *S. cerevisiae* RNase H2 **(Figure 1-7)**. Therefore, it is possible to characterize some of the AGS mutations in RNase H2 using yeast system.

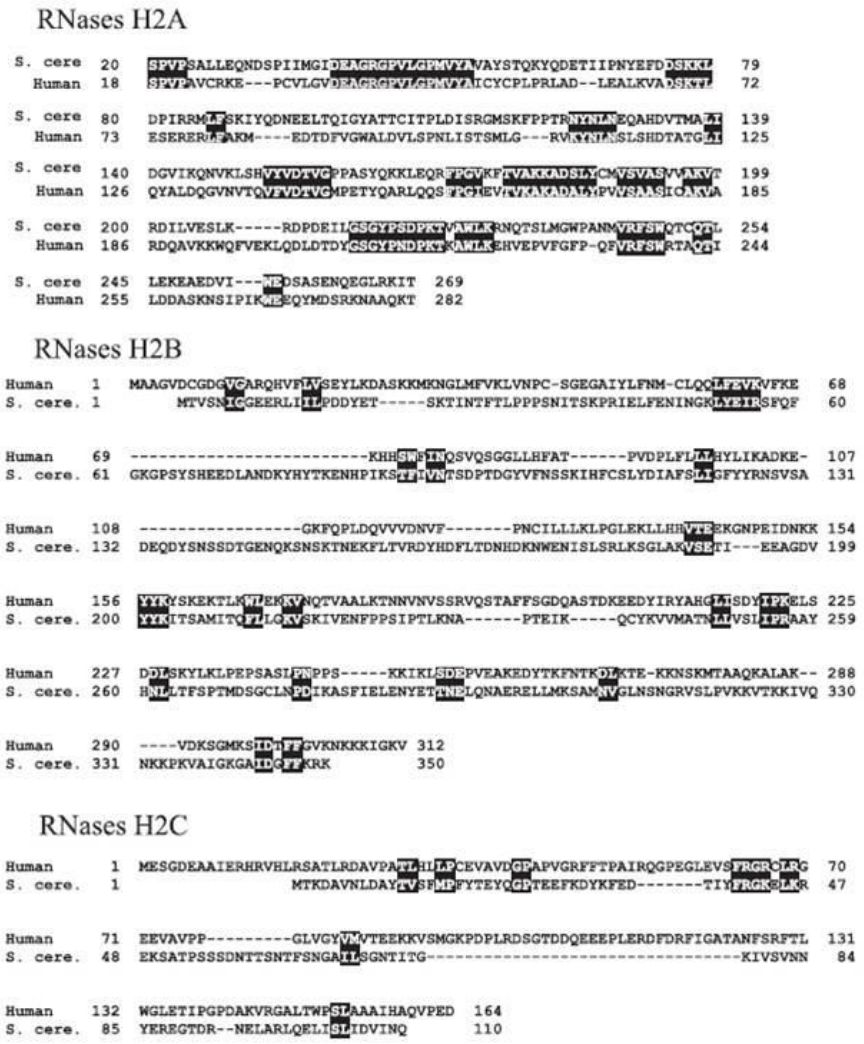

Figure 1-7. Comparison of conserved amino acid sequences of human and *S. cerevisiae* RNase H2 subunits. Adapted from Cerritelli and Crouch (2009) [13].

1.5 RNA-DNA HYBRIDS

In cells, RNA-DNA hybrids are readily found in a variety of processes including R-loops during transcription, replication of telomeres, hydrolysis of Okazaki fragment RNA primer, and RNA-driven repair [20, 21, 22, 23, 24]. Moreover, RNA-DNA hybrids can also form extra-chromosomally as intermediate structures during reverse transcription and generation of cDNA [25]. In yeast *S. cerevisiae*, transposons of yeast (Ty) synthesize cDNA by reverse transcription, some of which is transported into the nucleus and is integrated into the genome [26]. This process refers to retrotransposition.

1.6 TY1 RETROTRANSPOSONS

Retrotransposons are transposable elements that can transfer or copy their DNA by reverse transcription within a cell. Generally, a large number of eukaryotes have various types of transposons. Retrotransposons utilize reverse transcriptase to generate cDNA to be integrated into the genome for retrotransposition. Ty1 retrotransposon is one of the five transposable elements found in *S. cerevisiae* and affiliated with long terminal repeat (LTR) retrotransposons, which are located on both ends of the element. Ty1 retrotransposons is the most highly repeated Ty element in laboratory strains of *S.* cerevisiae, and nearly 25-35 copies of Ty1 elements present per haploid genome. Interestingly, Ty1 elements have similarities with retroviruses in terms of genome organization and replication [27]. Ty1 elements consist of two genes: *TY1A*, and *TY1B*, which are called *gag* and *pol* genes of retroviruses, respectively **(Figure 1-8)** [26].

Figure 1-8. Genome organization of Ty1 element in *S. cerevisiae*. It has detla (δ) LTRs at the ends of the element. (PR: protease, IN: integrase, RT: reverse transcriptase, and RH: RNase H).

TY1A proteins form a circle shape of structure with virus-like particles (VLPs). TY1B protein is divided into four different domains that are protease, integrase, reverse transcriptase, and RNase H. Ty1 elements and retroviruses amplify their DNA in a similar way. However, because Ty1 elements do not have *env* gene, which has an infiltration ability to target membranes, they are not infective. Retrotransposition of Ty1 elements is initiated with transcription of the elements **(Figure 1-9)**. The retrotransposition of Ty1 element is temperature sensitive. The optimal temperature of the retrotransposition is 22 ℃, and it is possible to detect at 30 ℃, but over 34 ℃ because at high temperature, inhibited processing of the TY1A/1B polyprotein and reduced reverse-transcriptase activity occur [28].

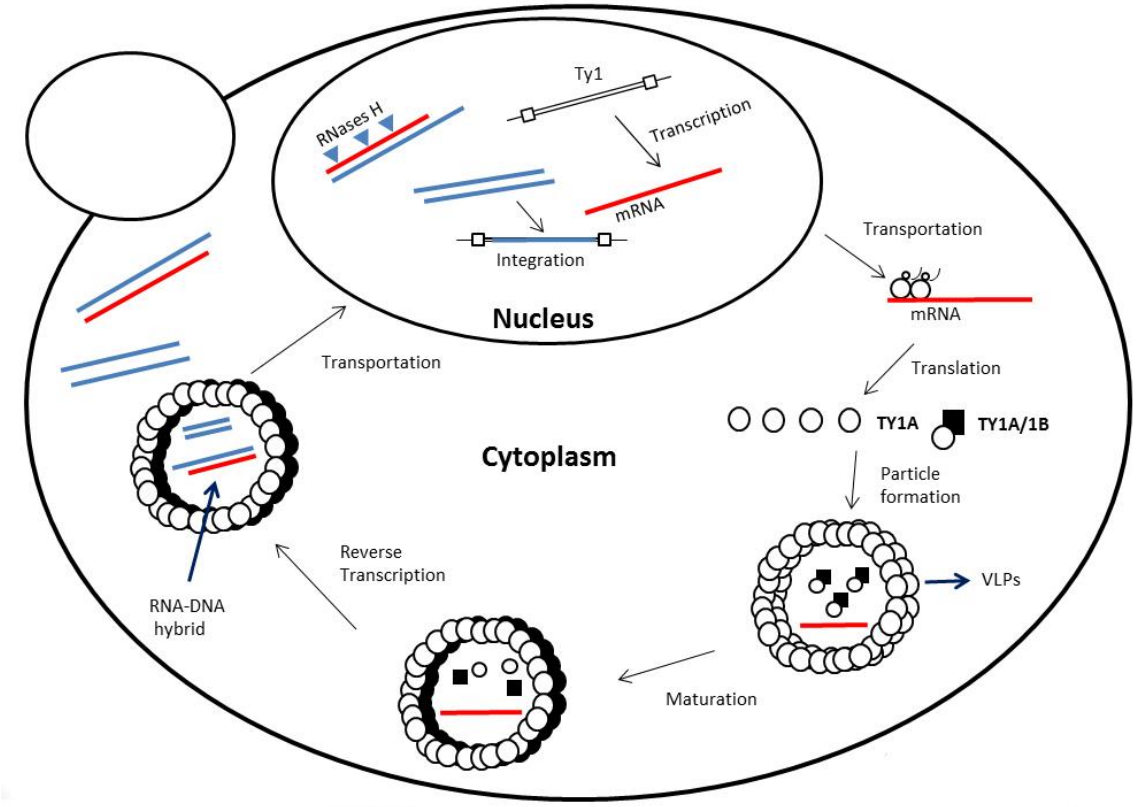

Figure 1-9. Retrotransposition cycle of Ty1 retrotransposons in *S. cerevisiae.* The transcribed Ty1-mRNA is transported to the cytoplasm to be translated into TY1A and TY1A/1B complex proteins. The Ty1-mRNA and TY1A/1B complex proteins are packed together within the VLPs, and subsequently TY1A/1B proteins are cleaved by protease. The synthesized cDNA from the Ty1-mRNA is randomly transferred into the nucleus to be integrated into the genome by integrase. Adapted from Krastanova, et al. (2005) [26].

1.7 RESEARCH GOALS & SIGNIFICANCE

We hypothesized that increased stability of extra-chromosomal RNA-DNA hybrid structures due to defects in RNases H could be a main cause to stimulate the autoimmune system in AGS. If this hypothesis is correct in RNases H defective cells, there should be abundant RNA-DNA heteroduplexes, such as those generated by reverse transcription of active retrotransposons in human cells. Because yeast cells also have retrotransposons, we aimed to test whether defects in RNases H affects Ty1 retrotransposition activity in yeast. The aims of the current study are:

1. Setting up and testing suitability of a retrotransposition assay in our cells
    - We planned to set up a Ty1 retrotransposition assay, originally designed by Curcio and Garfinkel in our yeast strains. We planned to perform the Ty1 retrotransposition assay both in the absence and in the presence of Ty retrotransposition suppressing factors and test its functionality.
2. Performing the retrotransposition assay with RNase H mutants including AGS-realated RNase H2 mutants
    - We planned to examine whether yeast cells with defective forms of RNases H and AGS-orthologous mutants of RNase H2 had altered retrotransposition activity compared with cells with wild-type RNases H.

The findings obtained from this study would help to reveal the unknown mechanism of the AGS associated with RNases H. In addition, this study could be helpful in future development of treatments for AGS patients.

CHAPTER 2

MATERIALS AND METHODS

2.1 PLASMIDS

The core plasmid in the study was pBDG598 **(Figure 2-1)**. This plasmid is also known as pGTy1mhis3-AI [29, 30]. The pBDG598 plasmid was introduced into in the yeast strains of the wild-type, RNases H-null and AGS-orthologous mutants. Once the *GAL1* promoter is induced by galactose, a pre Ty1-mRNA structure including Ty1 gene and *his3* gene interrupted by an artificial intron (AI) is produced. By processing, the AI is spliced out, and the *HIS3* gene is restored in the RNA script. Once the synthesized cDNA by RT is integrated by retrotransposition into the genome, His^+ colonies are detectable on SC-His^- medium.

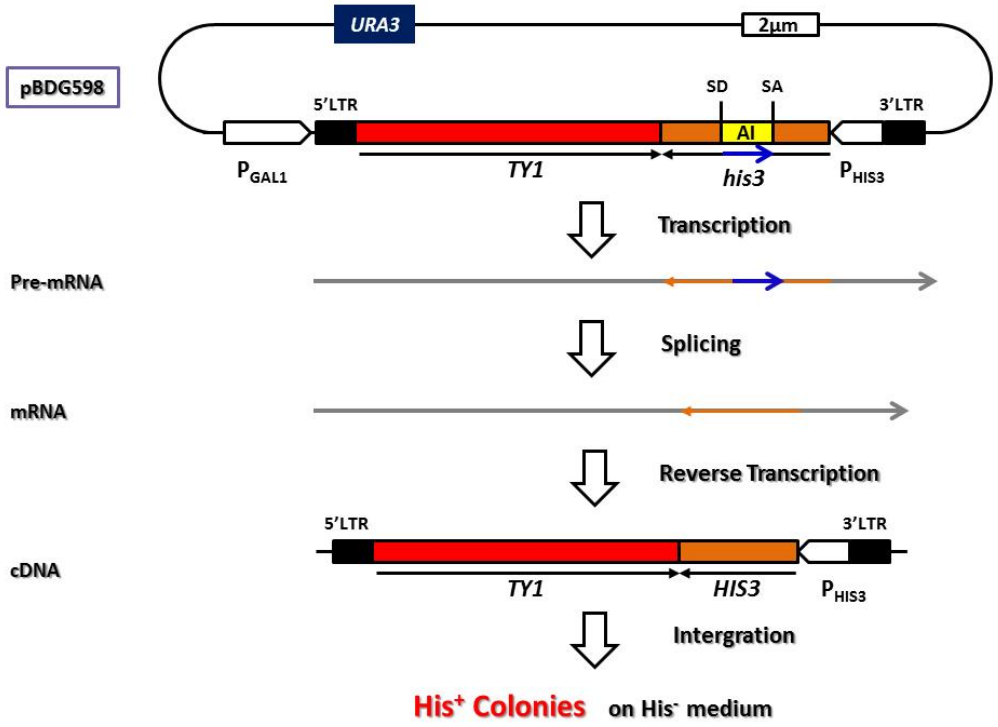

Figure 2-1. A diagram of plasmid pBDG598 used in this study. *URA3* is used as the main marker of the plasmid. Under the control of *GAL1* promoter, Ty gene is expressed.

The artificial intron (AI) inserted in the middle of *his3* gene is in the antisense orientation relative to the *HIS3* gene. Adapted from Derr and Starthern (1993) [30].

We also transformed pSM50 and pBDG102 plasmids into our strains **(Figure 2-2)**. The pSM50 plasmid has the *GAL1* promoter and disrupted *his3* gene but Ty1 element. Consequently, the restored *HIS3* cannot be integrated into the genome by retrotransposition, but it could happen with homologous recombination or other mechanisms [30]. From the strains including the pBDG102, any HIS^+ colony is not observed on SC-His^- medium as the plasmid has only the *GAL1*promoter.

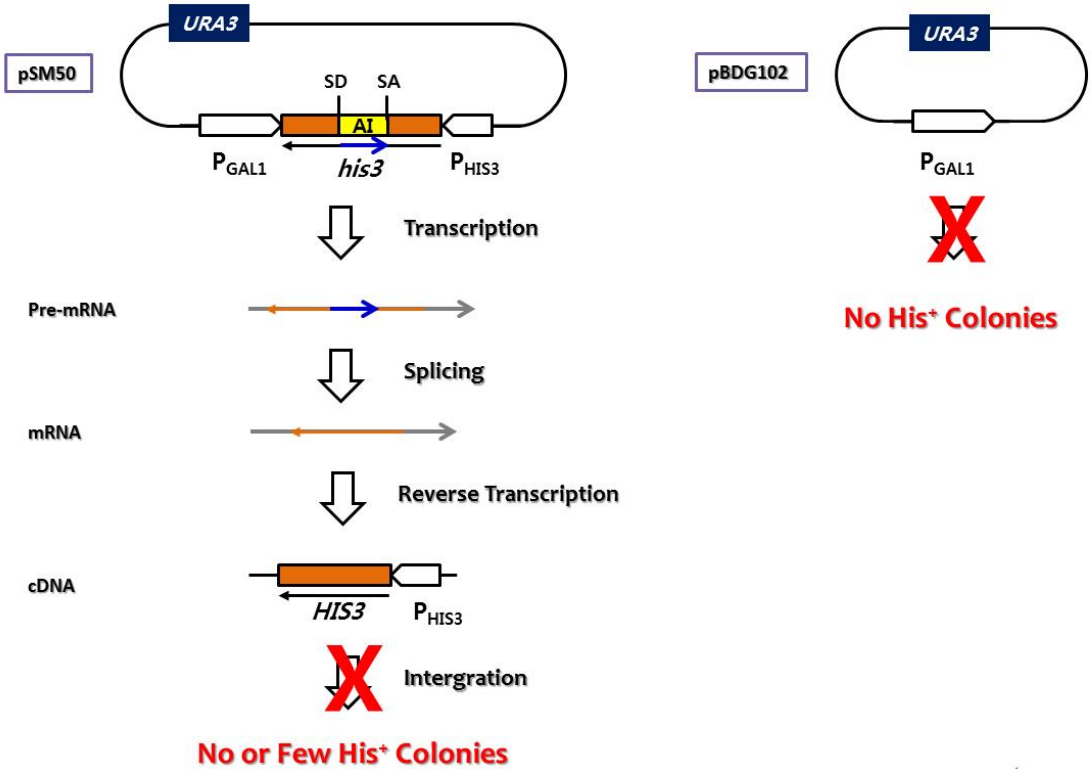

Figure 2-2. A diagram of pSM50 and pBDG102 plasmids used for negative controls in the study. (A) pSM50: due to the lack of Ty gene including an integrase domain, the *his3* gene interrupted by the AI cannot be restored. However, some of the yeast strains often show His^+ colonies on SC-His^- medium by possibly homologous recombination or unknown mechanisms. (B) pBDG102: no gene exists under the downstream of *GAL1* promoter. Adapted from Derr and Starthern (1993) [30].

2.2 STRAINS

TABLE 1. Yeast Strains used in this study.

Strain	Genotype	Plasmid	Source
TY 1-1, 1-2	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	pBDG102	this study
TY 2-1, 2-2	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	pBDG598	this study
TY 3-1, 3-2	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	pSM50	this study
TY-17, 53	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5*	pBDG598	this study
TY-18, 56	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5 rnh201Δ::kanMX4*	pBDG598	this study
TY-19, 58	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5 rnh201-G42S*	pBDG598	this study
TY-20, 61	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5 rnh203-K46W*	pBDG598	this study
TY-21, 64	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5 rnh1Δ::hygMX4*	pBDG598	this study
TY-22, 67	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5 rnh1Δ::hygMX4 rnh201Δ::kanMX4*	pBDG598	this study
TY-23, 70	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5 rnh201-G42S rnh1Δ::hygMX4*	pBDG598	this study
TY-24, 73	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5 rnh203-K46W rnh1Δ::hygMX4*	pBDG598	this study
TY-32, 52	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5*	pBDG102	this study
TY-33, 57	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5 rnh201-G42S*	pBDG102	this study
TY-34, 60	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5 rnh203-K46W*	pBDG102	this study
TY-35, 63	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5 rnh1Δ::hygMX4*	pBDG102	this study
TY-36, 66	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5 rnh1Δ::hygMX4 rnh201Δ::kanMX4*	pBDG102	this study
TY-37, 69	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5 rnh201-G42S rnh1Δ::hygMX4*	pBDG102	this study
TY-38, 72	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5 rnh203-K46W rnh1Δ::hygMX4*	pBDG102	this study
TY-55, 75	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5 rnh201Δ::kanMX4*	pBDG102	this study
TY-126. 127	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5*	pBDG102	this study
TY-128, 129	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5*	pBDG598	this study
TY-130, 131	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp5*	pBDG598	this study

S. cerevisiae strains used in the study.

As explained in Figure 1-7, we constructed AGS-related mutants in *S. cerevisiae* by amino acid substitution, using the similarity between human and yeast RNase H2 subunits **(Figure2-3)**. The G37S and R69W in human RNase H2 were converted to the G42S and K46W in yeast RNase H2, respectively.

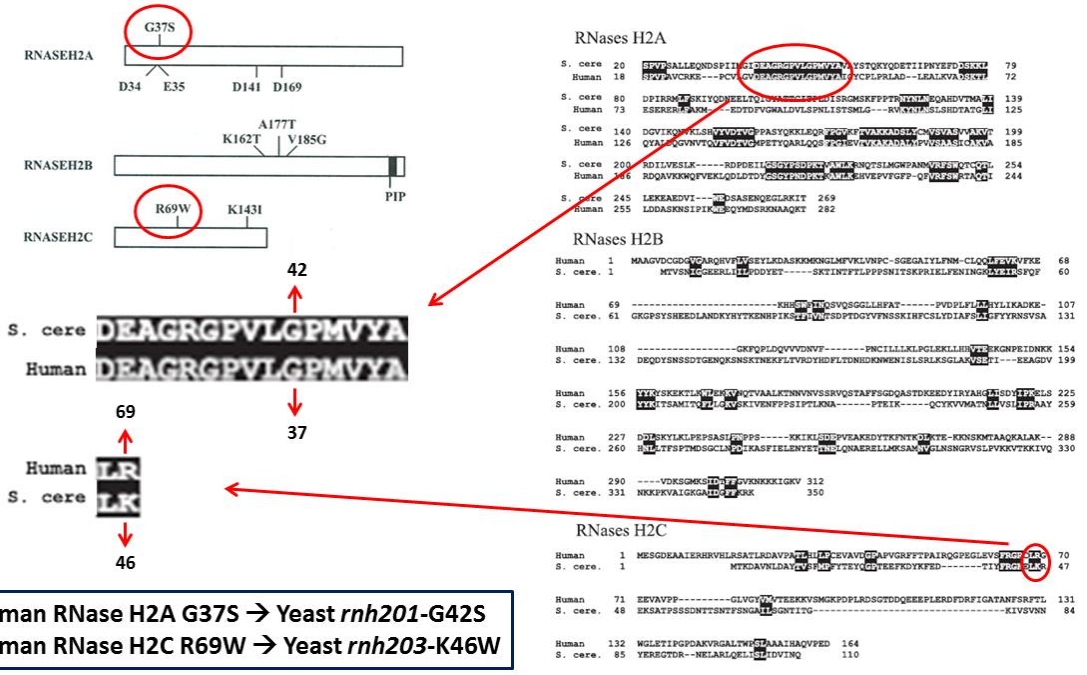

Figure 2-3. Construction of AGS-related mutant strains in yeast *S. cerevisiae*. (G: glycine, S: serine, R: Arginine, W: Tryptophan, and K: Lysine). Adapted from Cerritelli and Crouch (2009) [13].

2.3 SCHEME OF PATCHING ASSAY

Replica plate Ura^+ patches onto SC-His^- medium and SC-Ura^- medium with 2% galactose and incubate at 22 ℃ (96 hr) or 30 ℃ (48 hr), as indicated **(Figure 2-4)**. Then, replica plate again onto SC-His^- medium.

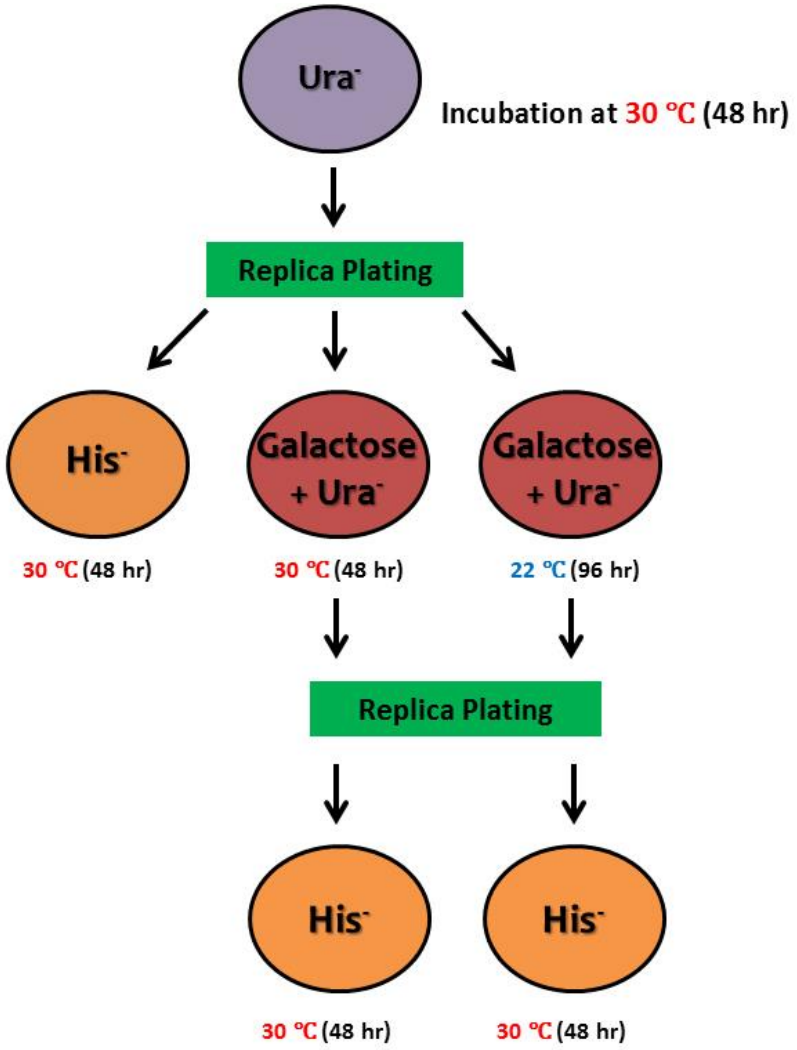

Figure 2-4. Steps of retrotransposition patching assay.

2.4 SCHEME OF LIQUID ASSAY

Inoculate strains in SC-Ura$^-$ liquid medium and incubate at 30 ℃ overnight. Transfer 10^6 cells into SC-Ura$^-$ liquid medium and SC-Ura$^-$ liquid medium with 2% galactose and incubate at 22 ℃ (96 hr) or 30 ℃ (48 hr), as indicated in the figure. Then, plate on SC-Ura$^-$ and SC-His$^-$Ura$^-$ media to detect His$^+$ colonies **(Figure 2-5)**.

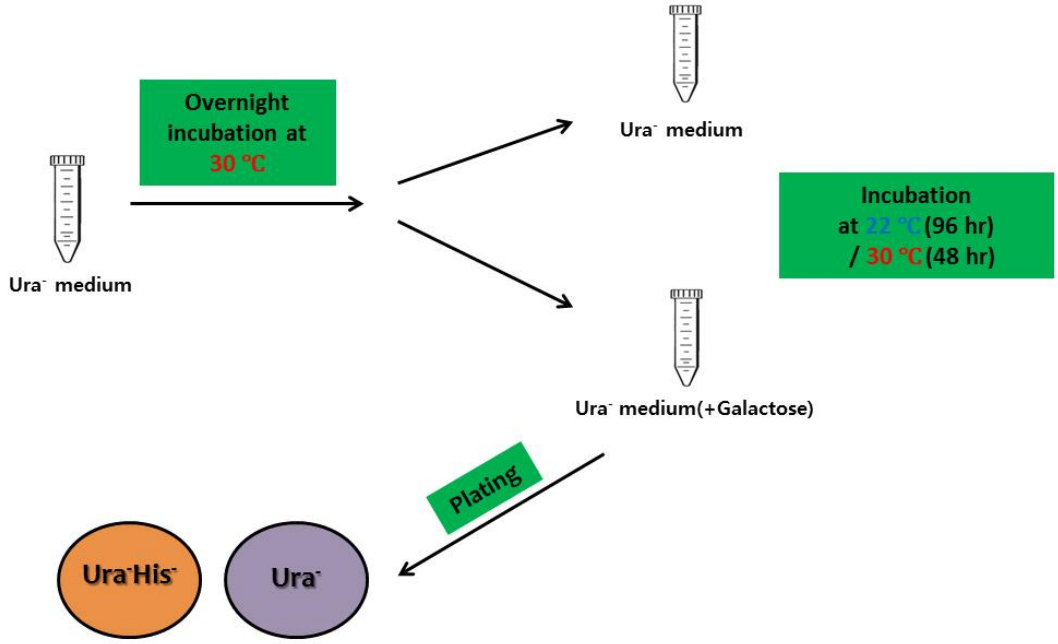

Figure 2-5. Steps of retrotransposition liquid assay.

CHAPTER 3

RESULTS

3.1 CONSTRUCTION OF RNASES H-NULL AND AGS-RELATED MUTANT STRAINS CONTAINING TY1 ELEMENT

We previously generated defective forms of RNase H mutant strains in yeast *S. cerevisiae* by replacing RNase H genes with antibiotic cassettes (*rnh1::hygMX4* and *rnh201::kanMX4*). In addition, as shown in Figure 2-3 [29], we constructed a couple of AGS-related mutant strains in yeast (*rnh201*-G42S and *rnh203*-K46W). From the AGS-related mutant strains, we also knocked out *RNH1* (*rnh201*-G42S *rnh1*::*hygMX4* and *rnh203*-K46W *rnh1*::*hygMX4*). To observe Ty1 retrotrasposition activity from these strains, we introduced a plasmid, pBDG598, which expresses the Ty1 retrotransposon fused to an inverted copy of the *his3* gene interrupted by an artificial intron (AI), which is in the antisense orientation relative to the *his3* gene, and the Ty1 element and the fused *his3* gene are expressed by the *GAL1* inducible promoter **(Figure 2-1)**. As negative controls, pBDG102 and pSM50 plasmids, which lack the Ty1 element **(Figure 2-2)** [29], were also introduced into the strains. Both of the pBDG102 and pSM50 have the *GAL1* promoter in common, but only pSM50 has the disrupted *his3* gene. As the three plasmids include an *URA3* gene as a marker, we initially screened and made Ura^+ patches of the following strains, including RNases H wild-type and double RNases H mutant *rnh1 rnh201,* on SC-Ura^- medium.

3.2 THE RETROTRANSPOSITION ASSAY WORKS IN OUR STRAINS

To test if the retrotranposition assay works in our yeast strain, we performed retrotransposition patching assay with the wild-type RNases H, containing pBDG102 (negative control NC), pBDG598 (Ty plasmid), pSM50, respectively **(Figure 3-1)**. Each of the strains was patched onto SC-Ura$^-$ medium. The Ura$^+$ patches were then replica plated onto glucose SC-Ura$^-$ (Ura$^-$) or galactose SC-Ura$^-$ (GalUra$^-$) media to see the difference between results when the *GAL1* promoter is induced and when it is not. The patched cells incubated at 22 ℃ for 96 hr or at 30 ℃ for 48 hr were then replica plated onto SC-His$^-$ (His$^-$) medium to observe His$^+$ colonies due to the retrotransposition of the Ty1 fused to the *his3* gene. We found that no His$^+$ colonies were generated from the strains containing pBDG102 without the Ty1 element, even in the presence of activated *GAL1* promoter **(Figure 3-1A, B, and C)**. In contrast to NC, the wild-type strains containing the Ty plasmid and pSM50 showed few His$^+$ colonies on His$^-$ medium in the absence of galactose likely due to the leaky nature of the *GAL1* promoter. When the *GAL1* promoter was induced, we observed high retrotransposition frequencies only for the strains containing the Ty plasmid compared to the strains containing the control plasmid **(Figure 3-1B, and C)**. As explained earlier, because high temperature down-regulates the processing of *TY1A/1B* protein and reverse transcriptase activity [28], more His$^+$ colonies were observed on the His$^-$ medium, when cells were replica plated from the GalUra$^-$ medium and incubated at 22 ℃. Overall, these findings ensured that our retrotransposition assay is applicable to detect the retrotransposition activity of the Ty1 element in our yeast strains.

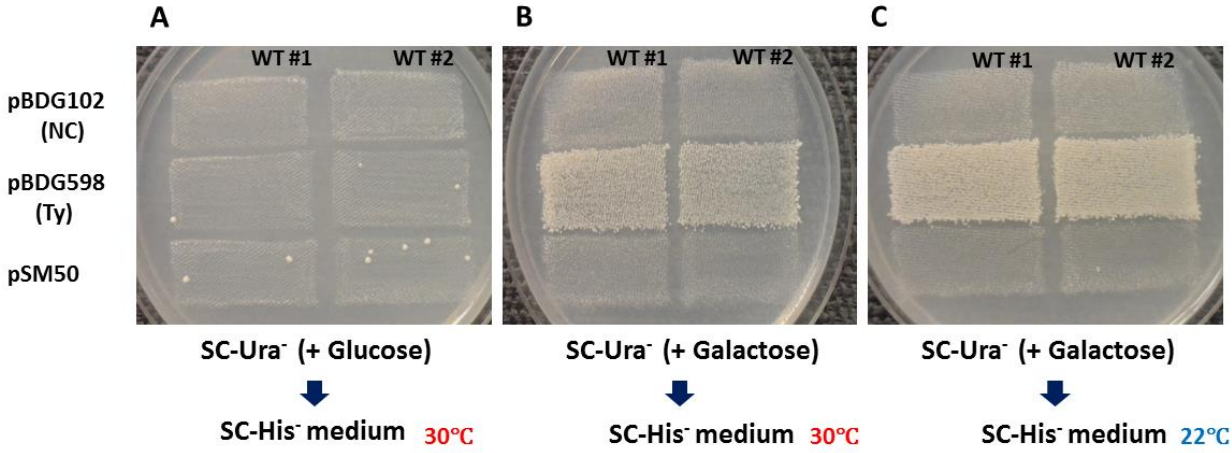

Figure 3-1. Result of retrotransposition patching assay with the wild-type. Replica-plated Ura^+ patches from (A) SC-Ura^- **(**+ Glucose), (B) SC-Ura^- **(**+ 2% Galactose), incubated at 30 ℃ and (C) SC-Ura^- **(**+ 2% Galactose), incubated at 22 ℃, to SC-His^- medium. (NC: negative control, Ty: Ty plasmid).

3.3 PHOSPHONOFORMATE (PFA) STRONGLY REDUCES THE TY1 RETROTRANSPOSITION

We tested our retrotransposition assay with Phosphonoformate (PFA), a reverse transcriptase inhibitor [33], to confirm that the His^+ colonies found on the His^- medium come from the outcome of the Ty1 retrotransposition. The Ura^+ patches of the wild-type strains were replica plated onto Ura^- or $GalUra^-$ medium with none, 1.0 mg/ml, and 2.5 mg/ml of PFA, respectively. Then, the patches were replica plated onto His^- medium once again after galactose induction. We found that the Ty1 retrotransposition was completely inhibited by PFA when cells were incubated at 30 ℃ **(Figure 3-2C and D).** Similarly, very few of His^+ cells grew on the His^- medium when cells were incubated at 22℃ **(Figure 3-2G and H)**.

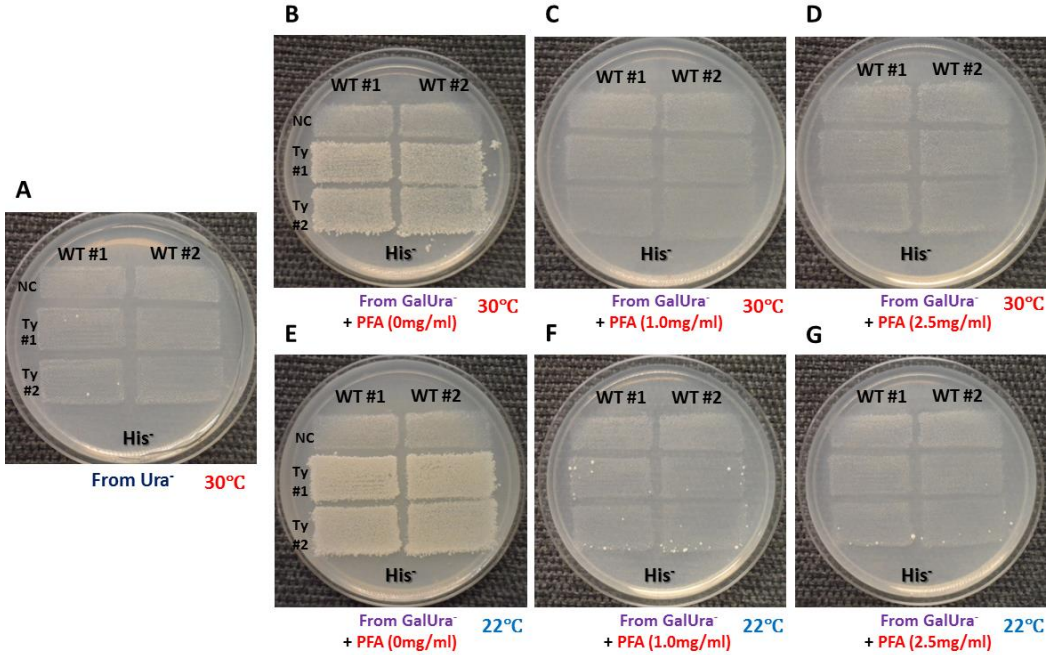

Figure 3-2. Result of retrotransposition patching assay with the wild-type in the presence of PFA. Replica-plated Ura^+ patches from (A) Ura^- medium in the absence of PFA, (B-D) $GalUra^-$medium in the presence of PFA as indicated, incubated at 30 ℃, (E-G) $GalUra^-$ medium, incubated at 22 ℃ to His^- medium.

To verify and quantify the results shown in the patching assay, we also performed the retrotransposition liquid assay with our strains **(Figure 3-3)**. The Ura^+ patches of the wild-type strains were initially incubated in Ura^- liquid medium at 30 ℃ for 24 hr. Then, 5ml of the medium was transferred into new Ura^- or $GalUra^-$ medium, and grew at 22 ℃ for 96 hr or at 30 ℃ for 48 hr. Later, cells were plated on His^-Ura^- and Ura^- media. The number of the His^+ cells grown in $GalUra^-$ liquid medium with PFA (1.0 mg/ml) was reduced about 15-fold compared to that grown in $GalUra^-$ medium without PFA at 30 ℃ **(Figure 3-3A)**, and when cells were incubated at 22 ℃, more than 1,500-fold **(Figure 3-3B)**.

Based on the results that retrotransposition activity was weakened in the presence of the reverse transcriptase inhibitor PFA, we concluded that the PFA strongly represses *HIS3* cDNA synthesis resulting in retrotransposition of the Ty1 element in our yeast strain background by inhibiting the reverse transcription process.

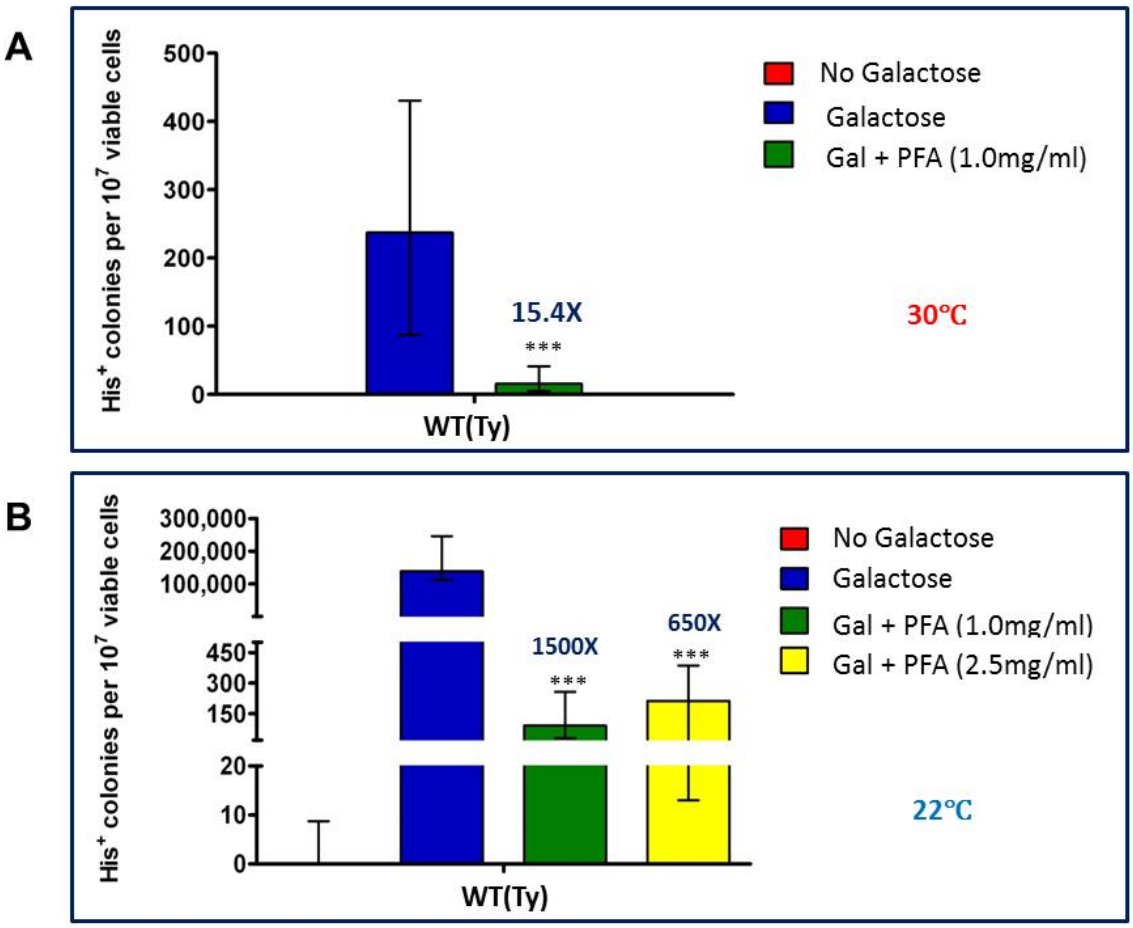

Figure 3-3. Result of retrotransposition liquid with the wild-type in the presence of PFA. The amount of grown His^+ cells in liquid media in the presence or absence of PFA incubated (A) at 30 ℃ or (B) at 22 ℃. The bars indicate the median values with range. The significant fold change is indicated on the top of the bars. The significance of comparisons between the strains was calculated using the Mann-Whitney U test * significant at $p \leq 0.05$; ** significant at $p \leq 0.01$. ($n = 6$).

3.4 RETROTRANSPOSITION IS PARTIALLY DECREASED IN THE ABSENCE OF DBR1 ENZYME

Dbr1 is an RNA lariat debranching enzyme that cleaves the 2'-5' phosphodiester linkage at the branch point of lariat intron pre-mRNAs after splicing. And then, it converts them into linear molecules that are subsequently degraded [34]. A study of Chapman (1991) revealed that *dbr1*-null mutant in yeast cells impairs cDNA formation and diminishes Ty transposition up to 10-fold [34]. We further confirmed that our retrotrasposition assay results arose from cDNA synthesized during reverse transcription by knocking out the *DBR1* gene in our yeast background. Retrotransposition frequency comparison between the wild-type and *dbr1* knocked-out strains was not clearly distinguishable by looking at the patches grown on the His^- medium and incubated at 22 ℃ **(Figure 3-4C)**. However, we found the number of His^+ colonies from *dbr1*-null strains to be significantly less than that of the wild-type shown on the His^- via the GalUra-, incubated at 30 ℃ **(Figure 3-4B)**.

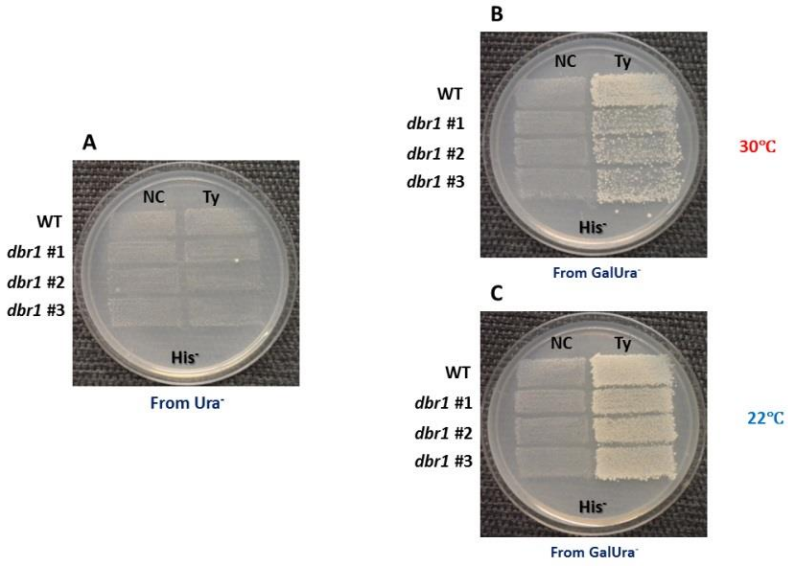

Figure 3-4. Result of retrotransposition patching assay with the wild-type and *dbr1* mutant strains. Replica-plated Ura^+ patches from (A) Ura^-, (B) $GalUra^-$ incubated at 30 ℃, and (C) $GalUra^-$ medium incubated at 22 ℃.

We also obtained a similar result from the liquid assay in the absence of the Dbr1 **(Figure 3-5)**. In the *dbr1* cells, a 9.1 fold-decrease in the amount of the His^+ cells was detected from the result of the retrotransposition liquid assay performed at 22 ℃ **(Figure 3-5B)**. At the 30 ℃ experiment, we also observed the reduced retrotransposition frequency between the wild-type and the *dbr1* mutant strains, but modest **(Figure 3-5A)**.

To sum up, we found that deletion of *DBR1* partially reduces retrotransposition activity shown in the Chapman (1991) study.

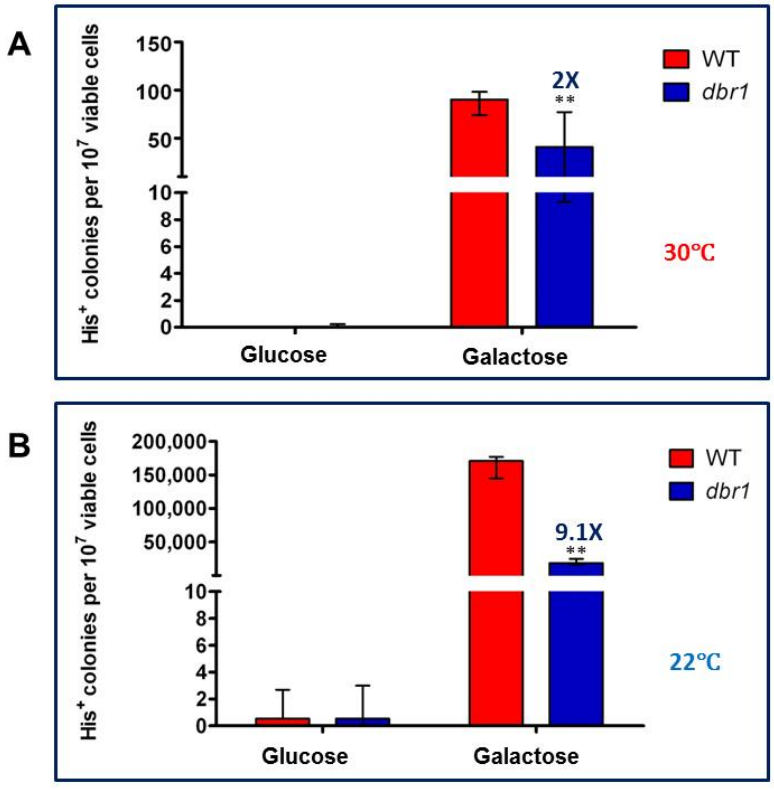

Figure 3-5. Result of retrotransposition liquid assay with the wild-type and *dbr1* mutant strains. The amount of grown His^+ cells in liquid media in the presence or absence of the Dbr1 incubated (A) at 30 ℃ or (B) at 22 ℃. The bars indicate the median values with range. The significant fold change is indicated on the top of the bars. The significance of comparisons between the strains was calculated using the Mann-Whitney U test * significant at $p \leq 0.05$; ** significant at $p \leq 0.01$. ($n = 6$).

3.5 TY1 RETROTRANSPOSITION ACTIVITY IS REPRESSED BY THE DELETION OF RNASES H1 AND H2

We then examined how RNases H and AGS-related mutant strains affect the retrotransposition of the Ty1 element through our retrotransposition assay. Each time we performed the patching assay we found a few His^+ colonies for the *rnh1 rnh201* double knocked-out mutant strain even though the *GAL1* promoter was not induced **(Figure 3-6A)**. We assumed that this result could be retrotransposition or gene conversion due to the leakiness of the *GAL1* promoter. Because of the enhanced retrotransposition activity in galactose when cells were incubated at low temperature, we could not find any difference among the mutant strains compared to the RNase H wild-type strain **(Figure 3-6C)**. Differently, when cells were grown on $GalUra^-$ medium and incubated at 30 ℃, it was found that the retrotransposition frequency was reduced when RNase H2 was deleted alone, or it was deleted together with RNase H1 **(Figure 3-6B)**. The *rnh1*-null and AGS-orthologous mutant strains also showed slightly decreased retrotransposition frequencies but not as much as shown in the *rnh201* mutant strain.

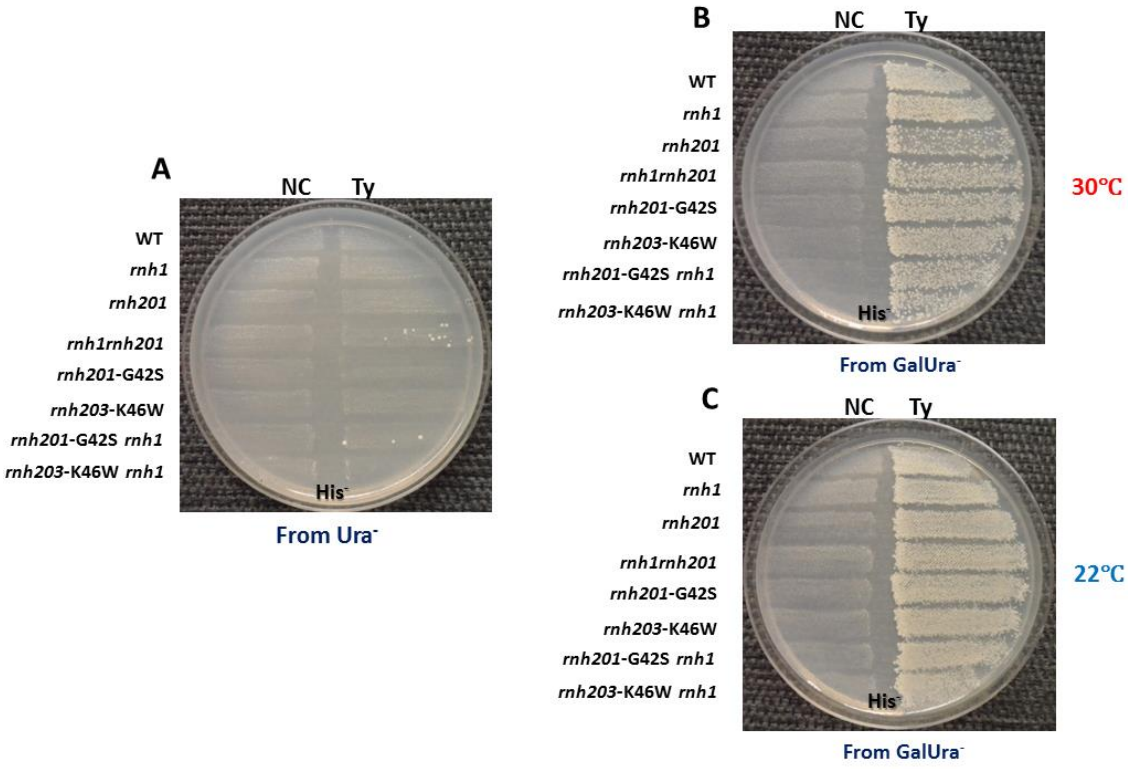

Figure 3-6. Result of retrotransposition patching assay with the wild-type, RNases H-null, and AGS-related mutant strains. Replica-plated Ura$^+$ patches from (A) Ura$^-$, (B) GalUra$^-$ incubated at 30 ℃, and (C) GalUra$^-$ medium incubated at 22 ℃.

To verify the patching assay result, we performed the liquid assay for the same strains. In the test conducted at 30 ℃ using defective forms of RNases H and AGS-related mutant strains, five out of seven mutant strains showed reduced number of His$^+$ colonies **(Figure 3-7A)**. While the *rnh1*, *rnh201*, *rnh201*-G42S *rnh1*, *and rnh203*-K46W *rnh1* strains partially showed decreased retrotransposition frequencies ranging from 1.2 to 3.2 folds as shown in the **Figure 3-7A**, the *rnh1 rnh201* double mutant reduced the retrotransposition frequency more than 11-fold compared to the wild-type. The result obtained from the 22 ℃ assay was fairly consistent with the patching assay result obtained at 30 ℃ **(Figure 3-7B)**. Only the *rnh201* mutant strain showed significantly a 10 fold-decrease of His$^+$ colonies

compared to the wild-type strain. In addition, *rnh1 rnh201* and *rnh201*-G42S *rnh1* knocked-out strains showed less than 3-fold reduced retrotransposition frequency compared to the wild-type strain.

From the result of both the patching and the liquid assays, *rnh201* single *and rnh1 rnh201* double mutants have a significant impact on the retrotransposition activity depending on the incubation temperature. The AGS-related mutant strains did not effectively increase or decrease the retrotransposition frequency of the Ty1 element, but when knocked out together with RNase H1, partially decreased the retrotransposition frequency in the liquid assay. The *rnh1* mutant strain showed a slight decreased number of His^+ colonies only in the liquid assay conducted at 30 ℃.

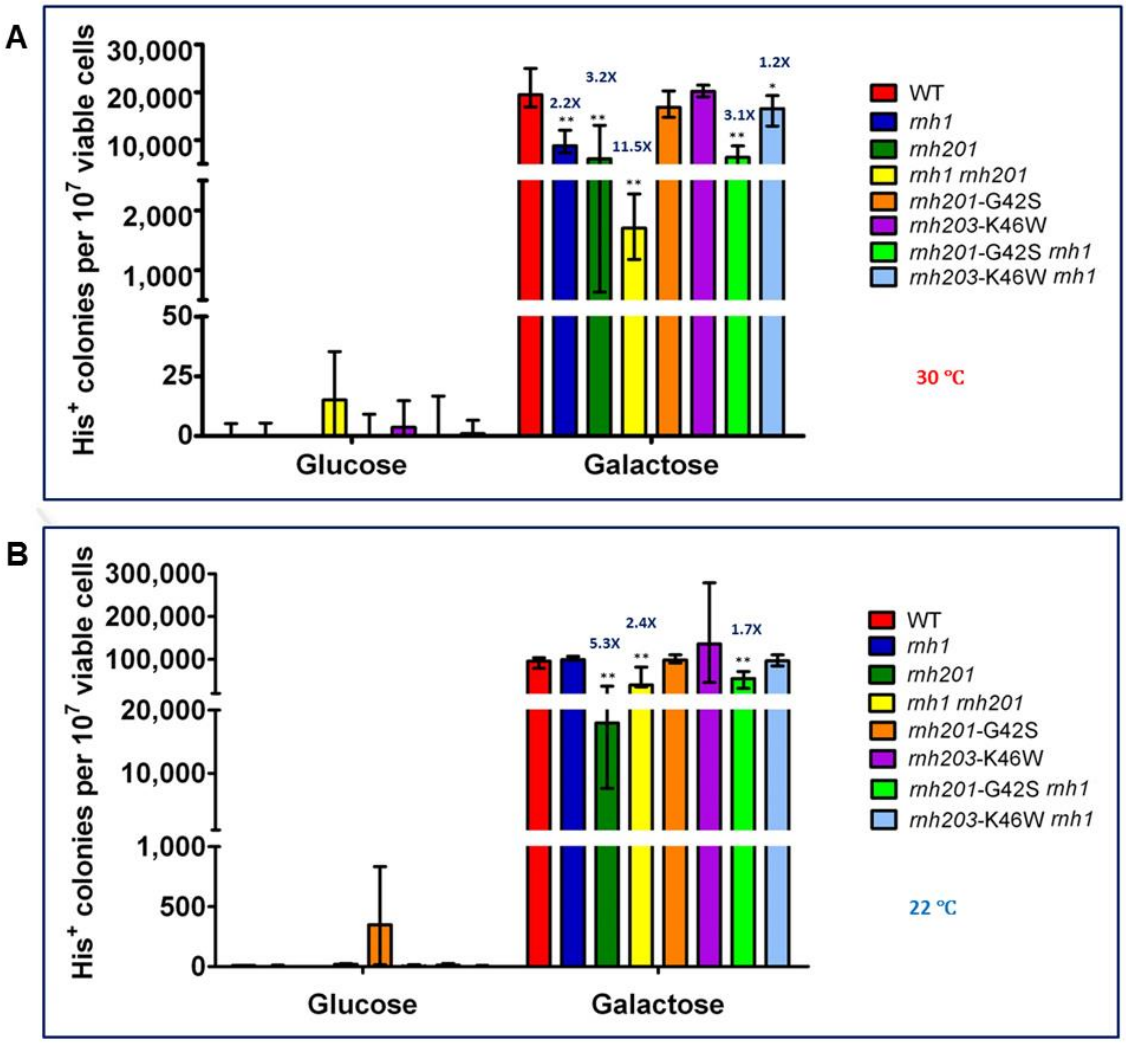

Figure 3-7. Result of retrotransposition liquid assay with the wild-type, RNases H-null, and AGS-related mutant strains. The amount of grown His^+ cells in liquid media incubated (A) at 30 ℃ or (B) at 22 ℃. The bars indicate the median values with range. The significant fold change is indicated on the top of the bars. The significance of comparisons between the strains was calculated using the Mann-Whitney U test * significant at $p \leq 0.05$; ** significant at $p \leq 0.01$. ($n = 6$).

CHAPTER 4

DISCUSSION

Aicardi-Goutières syndrome (AGS) is a rare genetic encephalopathy that mimics congenital viral infection characterized by increased interferon-α expression. It has been found that six gene are involved in AGS. These are TREX1, 3'→5' exonuclease, SAMHD1, cellular phosphohydrolase, ADAR1, RNA editing enzyme, and RNase H2 subunits, which are mutated in more than 50% of affected AGS patients. Mutations occurring in both types of RNase H, which cleave both isolated ribonucleotides embedded in DNA and RNA-DNA hybrids, can cause AGS. The mechanism of AGS is still unclear and no cure for AGS patients has been developed. It remains uncertain which function of RNase H2 is associated with AGS in RNase H2 mutants of AGS patients, and also there have been no scheme to detect mutation in RNase H1 could be associated with AGS. We hypothesized that incomplete work of RNase H2 could lead to accumulations of extra-chromosomal RNA-DNA hybrids generated during reverse transcription of retrotransposition. In this study, we tried to understand the relation between RNases H and yeast retrotransposition in the context of the AGS, using RNase-null and AGS-related mutant strains of *S. cerevisiae*.

Initially, we constructed some mutant strains that are defective forms of RNases H (*rnh1*, *rnh201*, and *rnh1 rnh201*). We also generated AGS-related mutant found in AGS patients (*rnh201*-G42S and *rnh203*-K46W)[13]. We then planned to set up a retrotranposition

assay using the Ty1 system developed by Curcio and Garfinkel [29] to detect Ty1 retrotransposition activity in our yeast strains. We transformed yeast cells with a recombinant DNA plasmid containing Ty1 retroelement fused to a *his3* gene, whose function is disrupted by an inserted artificial intron in the reverse orientation relative to the *his3* gene. The activation of the *GAL1* promoter upstream of the Ty1 element enables retrotransposition of the Ty1-*his3* fusion and then *HIS3* cDNA is synthesized through reverse transcription.

In order to test the functionality of the retrotransposition assay, we conducted the retrotransposition patching assay with the wild-type RNases H strains at two different temperatures (22 ℃ and 30 ℃). The results performed at both temperatures showed many numbers of His^+ colonies grown on His^- medium when the *GAL1* promoter was activated. Due to the improved protease and reverse transcriptase functions, more His^+ colonies were shown when the experiment was conducted at 22 ℃. In contrast, we did not observe any His^+ colonies from the strains with the plasmid lacking of Ty1 element. However, we found few His^+ colonies from the strains containing pBDG598 and pSM50, which contains the only the *GAL1* promoter and the *his3* gene, in the absence of galactose. This was thought to be because of the leaky nature of the *GAL1* promoter. These findings verified that the Ty1 retrotransposition assay works in our yeast strains.

We successively performed to test to verify the observed His^+ colonies where requiring reverse transcription step and a cDNA intermediate. In the first test, the wild-type RNase H cells were incubated in $GalUra^-$ medium containing phosphonoformic acid (PFA) inhibiting reverse transcriptase. The result shown from both the patching and liquid assays indicated that PFA significantly reduces Ty1 retrotransposition activity.

By knocking out the *DBR1* gene, which is required for efficient cDNA formation in yeast cells [34], we found that the Ty1 retrotransposition frequency is partially but significantly reduced in the absence of *DBR1* gene.

Having the established functionality of the Ty1 assay in our yeast strains, we decided to perform the retrotransposition assay in the RNase H-null and AGS-orthologous mutant strains. We found that *rnh201* and *rnh1 rnh201* strains showed reduced transposition frequency in the patching assay when cells were grown on GalUra$^-$ medium and incubated at 30 ℃. In the liquid assay performed at 30 ℃ or 22 ℃, we observed that most of the strains had decreased Ty1 retrotransposition frequency, except for the AGS-related mutants. Importantly, defects in RNase H1 and/or H2 did not increase the frequency of Ty1 retrotransposition. Differently, a decrease of the Ty1 retrotransposition frequency was apparently observed in *rnh201* and *rnh1 rnh201* cells and also in *rnh201*-G42S *rnh1* cells from the results of the liquid assays conducted at both temperatures, and a minor decrease of Ty1 retrotransposition frequency was observed in the *rnh1* mutant only in the test conducted at 30 ℃. Interestingly, most of the time the patching assay was done with the *rnh1 rnh201* double mutant strain at both temperatures, few of its His$^+$ colonies were observed without the *GAL1* promoter induction. We guessed it might occur as the *GAL1* promoter is leaky and the retrotransposition of Ty-*his3* could be in the absence of both types of RNase H because there should be more cDNA. A contradictive view on this, the *rnh1 rnh201* mutant apparently showed a decrease of Ty1 retrotransposition activity in the presence of galactose but reversely showed His$^+$ colonies in the absence of galactose. The few His$^+$ colonies without galatose induction in the *rnh1 rnh201* cells could be due to recombination between cDNA and *his3* alleles on the plasmid.

There are a couple of possible explanations why the Ty1 retrotransposition frequency is reduced in the absence of RNases H enzymes. First of all, substrate preference of the integrase binding to nucleic acids may delay or inhibit the Ty retrotransposition activity. The binding of the intergrase to DNA duplexes could not be selective due to the increased abundance of RNA-DNA substrate in RNase H defective cells. However, decreased amount of double-stranded DNA can force the integrase to automatically bind RNA-DNA hybrids instead of DNA duplexes. The change on substrate of the integrase might delay or inhibit the retrotransposition process. Furthermore, if RNA-DNA hybrid fragments were potentially integrated into the genome, it also would cause a problem on retrotransposition. The integrase may recognize RNA-DNA hybrids as a DNA duplex by capturing both pure DNA ends of the strands.

As part of future work, we plan to identify and quantify extra-choromosomal RNA-DNA hybrids of the *HIS3* gene from our RNase H defective strains either by Southern blotting or with the S9.6 antibody specific to RNA-DNA hybrids. Ultimately, we hope to apply our retrotransposition assay in human cell line, such as long interspersed element-1 (LINE-1), which is a retrotransposable element found in human to observe the retrotransposition activity in the defective forms of RNase H.

Our findings in this study could become a helpful source to unveil the mechanism how RNase H defects and to implement novel treatments for people suffering from the AGS.

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